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The two forms of the S_2 state multiline signal in Photosystem II: effect of methanol and ethanol

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Abstract

The characteristic Mn hyperfine 'multiline' signal exhibited in the S_2 state of the oxygen-evolving complex (OEC) complex of Photosystem II (PSII) has been shown to be heterogeneous in character. In this study, we have explored the effects that influence the proportions of the two forms of the S_2 state multiline signal present in any sample. The narrow form of the signal is lost upon storage (weeks) at 77 K, whereas the broad form remains. In particular, we explore the roles of ethanol and methanol as well as effects of the second turnover of the enzyme on storage of the sample at 77 K. We find that in samples containing methanol, the narrow form may predominate upon the first flash, but the broad form predominates on the fifth flash and also in samples containing ethanol.

Keywords: EPR; Manganese; Photosystem II; OEC; Multiline signal

1. Introduction

The oxygen-evolving complex (OEC) of Photosystem II (PSII) catalyses the oxidation of water to molecular oxygen. At the centre of the enzyme is the P680 chlorophyll complex that is oxidised when a quantum of light captured by the light-harvesting complex reaches it. Its electron is passed via a pheophytin molecule to Q_A, the first quinone acceptor. In order to ensure stable charge separation, $P680^+$ is quickly reduced by Y_Z, a tyrosine residue on the D1 peptide. Y_Z is in turn reduced by the OEC, which consists of four manganese ions, a calcium and a chloride ion and is thought to bind the substrate water required for O_2 evolution. The OEC is capable of storing, in a cyclic manner, four oxidising equivalents before it oxidises water to molecular oxygen in a concerted process and the cycle starts over. The intermediate oxidation states of the OEC are denoted $S_0 \ldots S_4$, where the suffix refers to the number of electron holes stored. The S_1 state is the dark stable state. The paramagnetic S_2 state can be formed either by continuous illumination at ~ 200 K or by laser flashes. One laser flash generates the S_2 state multiline, as does five laser flashes on the second turnover of the enzyme. The S_2 state multiline arises from the hyperfine interactions of at least two coupled Mn in a net $S = 1/2$ spin state. It has about 20 hyperfine lines and a g-value of about 1.98 [1]. Another broad featureless EPR signal, centred at $g = 4.1$ and attributed to a higher spin state of the Mn cluster, can also be produced in the S_2 state. Illumination by light in the near infrared region (NIR), has been shown to convert the S_2 state multiline signal to the $g = 4.1$ signal without further turnover of the enzyme [2]. Alcohol added to the sample buffer (typically $\sim 3\%$) favours the formation of the multiline signal at the expense of the 4.1 signal.

de Paula and Brudvig [3], when studying the effect of long- or short-term dark adaptation prior to freezing on the multiline signal formed by low temperature illumination, found that the multiline varied in shape: a conventional

Abbreviations: ANU, Australian National University; Cyt, cytochrome; EPR, electron paramagnetic resonance spectroscopy; ESEEM, electron spin echo envelope modulation; EtOH, ethanol; MeOH, methanol; NIR, near infrared; OEC, oxygen-evolving complex; P680, primary electron donor; PPBQ, phenyl-*p*-benzoquinone; PSII, Photosystem II; UCL, University College London; Y_Z, tyrosine residue D1-Y161

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multiline signal (with low-field hyperfine features) was formed when the membrane fragments were given 4 h of dark adaptation at 0 °C and then illuminated at 200 K. A narrower form of the multiline signal (lacking low-field hyperfine features) was formed when membrane fragments given a short-term (6 min) dark adaptation were illuminated at 160 K. The broad form also appeared when short-term dark-adapted membrane fragments were illuminated at 170 K or higher. Sivaraja et al. [4] found that the increase in magnetic susceptibility observed for the S_1-S_2 transition varied with the dark incubation time, supporting the notion of a resting and active state.

Boussac [5,6] found that only a portion of the multiline was sensitive to NIR. The amount varied between 20% and 70%, depending on buffer composition. After the NIR-induced conversion of the multiline to the $g=4.1$ signal, the remaining multiline was found to be narrower in spectral width than the conventional multiline signal. Such variations in multiline signals have been postulated and attributed to different spin states of the Mn cluster [7].

Lorigan and Britt [8] found biphasic relaxation behaviour for the multiline in short-term dark-adapted material, indicating the presence of two multiline signals, one with a greatly slowed spin-lattice relaxation.

Decay of the S_2 state multiline signal at 77 K has been observed in samples without exogenous electron acceptor [9]. Evans et al. [10] pursued electron spin echo envelope modulation (ESEEM) studies of membrane fragments poised in the S_2 state through 200 K illumination in the presence of methanol (MeOH) (3%, ~ 1 M) and 0.5 mM phenyl-*p*-benzoquinone (PPBQ). With [^{2}H]MeOH they observed ^{2}H modulation over a ~ 100 mT region in the centre of the multiline pattern, but not in the wings of the spectrum. When the sample was stored on liquid N_2 over a period of weeks, the ^{2}H modulation disappeared altogether. Concomitant with this change there was a loss of multiline signal. The resultant difference multiline signal was narrower than that which remained upon storage at 77 K. Both the multiline intensity and the methyl ^{2}H modulation were restored by re-illumination at 200 K. ESEEM studies in the presence of ^2H_2O showed a similar distribution of ^{2}H modulation and a narrower, more rapidly decaying multiline signal was also associated with this modulation [11]. ESEEM studies of methyl [^{2}H]MeOH interaction with the S_2 multiline have also been reported by Force et al. [12]. Both groups reported ^{2}H modulation specific to MeOH, consistent with the alcohol binding to the Mn cluster. Although a detailed analysis of the ^{2}H interaction with the multiline spin centre was not carried out in Ref. [10], the width of the frequency domain ^{2}H modulation peaks seen by Evans et al. suggest a much stronger hyperfine interaction than inferred by Force et al.

The above observations suggest that the multiline signal, as conventionally formed, may not be a homogeneous species. Here we have extended these studies to a comparison between the multiline signals seen after one and five flashes in the presence of small mono-alcohols in an attempt to explore these effects further.

2. Methods

2.1. Preparation of samples

PSII membrane fragments were prepared according to Ref. [13], frozen on liquid N_2 and stored at −80 °C until required, in a buffer containing 20 mM 2-(*N*-morpholino)ethanesulfonic acid at pH 6.0, 15 mM NaCl and 10 mM $MgCl_2$ and 400 mM sucrose. Oxygen activity was measured with a Clarke oxygen electrode. Typical O_2 activities for these membrane fragments were 800 (μmol of O_2) (mg of Chl)$^{-1}$ h^{-1}. Samples were prepared with or without 3% (v/v) MeOH or ethanol (EtOH) and PPBQ, 0.5 mM in dimethylsulfoxide. For X-band EPR experiments, samples were diluted to ~ 1.8 (mg of Chl) ml^{-1} in the same buffer. Pre-illumination and flash-freeze procedures were carried out as described previously [14]. PPBQ was added in the dark 1 min before the final flashes. Illumination was carried out using a Nd:YAG laser, giving 350 mJ per 7 ns flash at 532 nm. The samples were frozen immediately at 200 K and then transferred to liquid N_2 in readiness for EPR. PSII membrane fragments for Q-band samples were prepared in the dark at ~ 9 (mg of Chl) ml^{-1}, given no preflash, frozen to 190 K in a liquid N_2 flow cryostat and illuminated for 5 min with white light (2400 μmol of photons m^{-2} s^{-1}).

Samples used to obtain the spectra shown in Fig. 5 were prepared using the procedures in Evans et al. [10].

2.2. EPR spectroscopy

X-band (~ 9.4 GHz) EPR spectroscopy was carried out on a Bruker ESP300E spectrometer equipped with an Oxford He cryostat and temperature control system. Q-band (~ 34 GHz) studies were carried out on the same spectrometer equipped with an in-house modified Oxford He cryostat (R. Bramley, unpublished), allowing similar He flow rates and temperature stability as the X-band cryostat. Spectra were taken within 48 h (fresh) of the samples receiving the final flashes, or after 16 or more weeks (stored).

Double integration of the multiline signals for comparison of signal intensities was carried out using WinEPR software. The region containing contributions from Y_D was excised and the integration was over the same number of field points in each light-minus-dark spectra.

3. Results

3.1. Effect of small mono-alcohols

Fig. 1A shows the S_2 state multiline at X-band frequency with addition of MeOH (MeOH multiline) or EtOH (EtOH

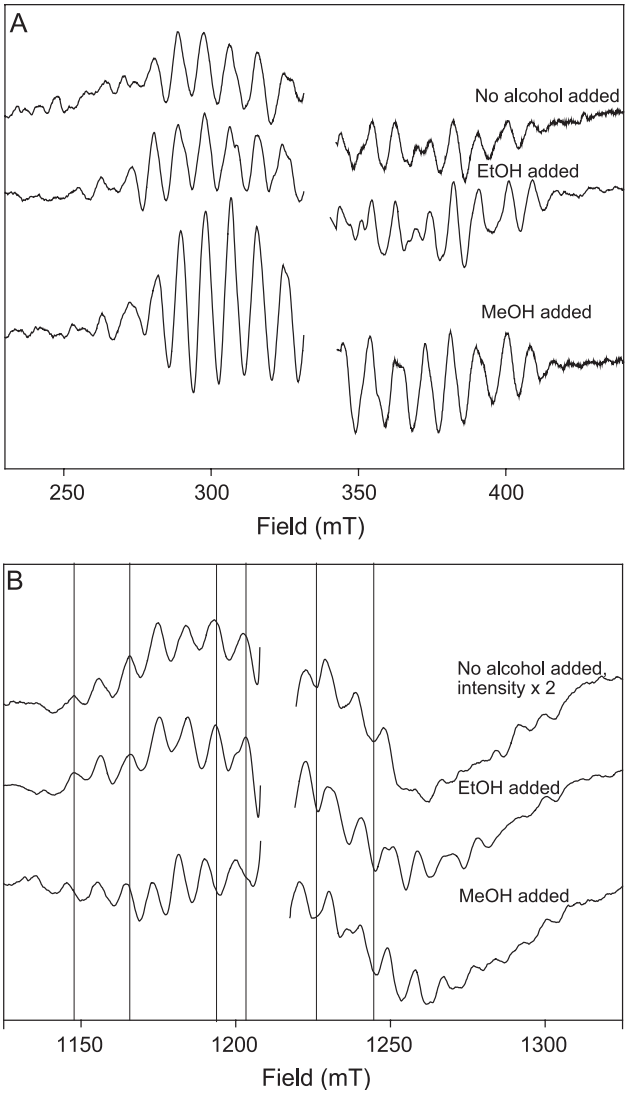

Fig. 1. X-band (A) and Q-band (B) EPR spectra of the S_2 state multiline signal in the presence of MeOH, EtOH and no alcohol. (A) Light-minus-dark spectra produced by single flash. Spectra acquired at 7 K, frequency 9.42 GHz; MF 100 kHz, MA 2 mT, MW power 6.32 mW. (B) Light-minus-dark Q-band spectra produced by continuous illumination. Spectra acquired at 9 K, frequency 33.97 GHz; MF 25 kHz, MA, 2 mT, MW power 1.9 mW.

multiline) and without alcohol, produced with one flash at room temperature. In these samples, there is no $g=4.1$ signal present as it decays rapidly at room temperature. All centres poised in the S_2 state are therefore in the multiline form. The spectrum of a sample with no additions and the EtOH multiline spectrum are very similar in intensity and overall line shape. The peak widths and positions are the same, but the EtOH multiline spectrum resolves more hyperfine structure within each major peak. The MeOH multiline, however, is quite different. The edges of this spectrum are overall similar to that of EtOH multiline, however, six peaks downfield of the $g=2$ region and at least four peaks upfield differ. They are sharper and the peak line width is narrower, contain no resolved hyperfine structure, but are about twice as intense as the corresponding peaks in the EtOH multiline spectrum. Because the peaks are narrower there is a slight shift in peak positions for the MeOH multiline spectrum and the spectrum appears contracted relative to the EtOH multiline spectrum.

Fig. 1B shows the multiline signals from samples with the same additions, poised in the S_2 state by 190 K illumination, at Q-band frequency (light minus dark). The signals in flashed samples have the same overall shape as the 190 K illuminated samples, but are less well-resolved. At Q-band frequency the Zeeman term is more dominant and the *g*-values of the signals are more easily resolved. It is immediately apparent that the multiline of the sample

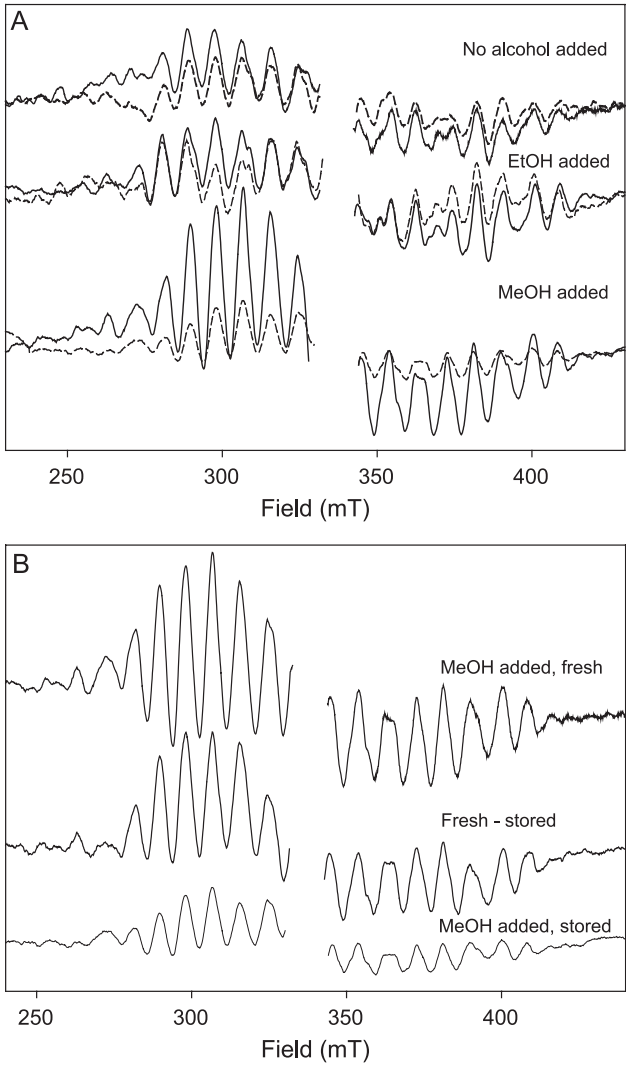

Fig. 2. (A) Effect of sample storage at 77 K on signal intensity, for light-minus-dark spectra produced by single flash, with MeOH, EtOH and no additions. The difference in signal intensity represents a loss ~ 20% loss for samples with no additions and EtOH and ~ 60% for samples with MeOH. (B) Details of one-flash sample with MeOH: spectrum taken of fresh sample (top), spectrum taken of sample stored 16 weeks at 77 K (bottom) and the difference between the two, forming the narrow multiline (middle). All spectra are difference spectra and acquired at 7 K, frequency 9.42 GHz; MF 100 kHz, MA 2 mT, MW power 6.32 mW.

Table 1
Summary of multiline signal intensity in samples with different alcohols, number of flashes, and storage time

Alcohol added	Relative intensity (%)[a]	five-flash ML/1-flash ML (%)	Amount of signal lost on storage (%)[b]		Relative amount of signal lost on storage (%)[c]
MeOH	100	62	one flash	60	60
			five flash	0–8	0–5
EtOH	60	60	one flash	17	10
No additions	56	60	one flash	23	14

[a] Integrated signal intensity relative to maximum intensity seen in MeOH samples.
[b] Reduction of signal intensity in a particular sample upon storage (16 weeks).
[c] Reduction of signal intensity from (b) relative to initial MeOH sample intensity.

without alcohol and the EtOH multiline again are very similar in overall shape and width, whereas the MeOH multiline shows some differences. These are mainly in the peak positions, which for the MeOH multiline spectrum are significantly shifted particularly near $g=2$, relative to the other spectra. There are two possibilities for this effect: superposition of two different signals or a change in the ligand environment of the Mn cluster in the presence of MeOH. Preliminary simulation studies indicate that superposition of two multiline signals at Q-band is unlikely to give the outcome observed here. The *g*-tensor change for the MeOH-containing sample is therefore indicative of a change in ligand environment, perhaps caused by MeOH itself binding to the Mn cluster. The effect is maintained in samples given five flashes and also maintained in samples stored for up to a year at 77 K (data not shown).

The Q-band MeOH multiline does not have the additional intensity observed in the X-band samples, when compared with the EtOH multiline. The spectra at Q-band also have an underlying signal of unknown origin. Both of these features may be due to *g*-strain, apparent at this frequency, although we cannot rule out that the underlying signal is an artefact. However, it is worth noting that the underlying signal shape in the MeOH sample is somewhat different to that of the EtOH sample and that with no additions. For the MeOH sample the underlying signal is shifted to lower *g*-value. The spectrum without any alcohol is much less intense at Q-band. These samples are generated by illumination at 190 K and this protocol co-generates some $g=4.1$ signal in samples without alcohol (not shown).

The Q-band data indicate that there is a change in the ligand environment for MeOH samples compared with EtOH and no additions, suggesting that MeOH itself is a ligand to the Mn.

3.2. Effect of storage of sample

The results from the above X-band studies raise the possibility that a second component may be present in the MeOH multiline. The S_2 state multiline was acquired in samples given one flash. The sample was then stored on liquid N_2 for several weeks as suggested in Evans et al. [10] and the multiline signal was again collected. The spectra taken fresh and after 16 weeks' storage are shown in Fig. 2A and the data summarised in Table 1. The loss in multiline signal in a sample stored for 16 weeks compared with the fresh sample represents about 20% in the EtOH sample and the sample with no alcohol added. For the MeOH sample, however, the loss in multiline upon storage was about 70%. It is interesting to note that for the MeOH multiline the difference is largely in the central peaks. The smallest peaks downfield are not lost during the storage. The difference spectrum therefore reveals that the signal lost during storage is a narrower multiline, shifted upfield relative to the signal that remains. Fig. 2B shows the various multiline signals in the presence of MeOH, i.e. fresh, remaining on storage, and lost upon storage. This narrower signal, representing the

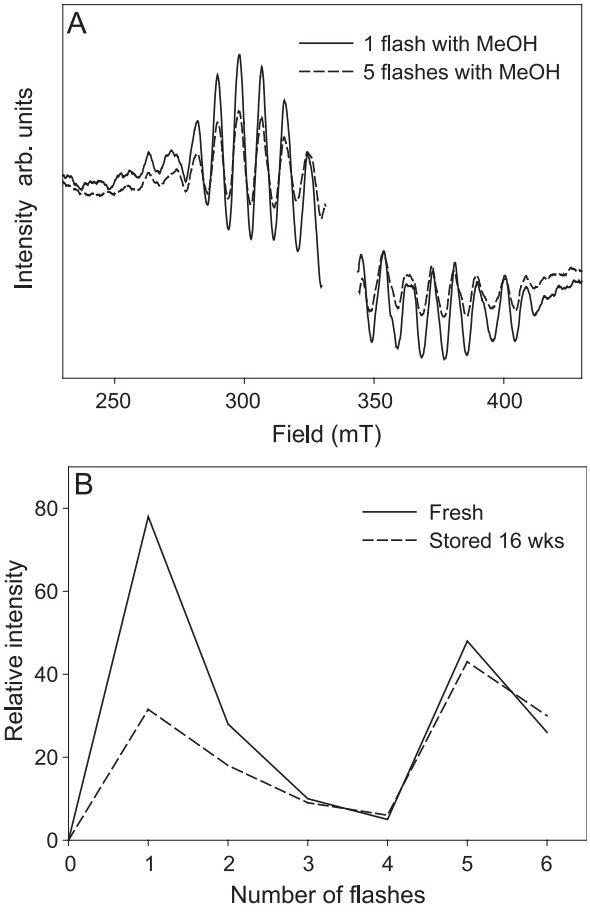

Fig. 3. (A) Comparison of the S_2 state multiline (difference) spectra after one and five flashes in a sample containing MeOH. The signal intensity of the five-flash spectrum is about 65% of the one-flash spectrum overall. Spectrometer conditions as in Fig. 2. (B) Oscillation of the S_2 state multiline signal with flash number, for a sample containing MeOH. The two curves represent spectra taken fresh and spectra taken after 16 weeks storage at 77 K. Signal intensity is estimated from three peaks downfield of $g=2$ such that contributions from other paramagnetic components are avoided (e.g. see Ref. [19]).

multiline lost upon storage, appears to contain about 16 Mn hyperfine lines, typical of dimeric Mn complexes.

The S_2 state multiline signal obtained after five flashes was also studied in the same manner for all sample types. Fig. 3A shows the MeOH one- and five-flash multiline spectra. After Kok analysis it is clear that the proportion of centres that form multiline after five flashes compared with the proportion forming multiline after one flash is about the same in all samples (Table 1). The S_2 state multiline after five flashes is overall about 62% of the one-flash multiline intensity, for multiline with no additions and with EtOH about 60%. However, in the MeOH multiline spectrum the edge peaks, particularly downfield, differ in intensity only by about 20% between the one-flash and five-flash samples. This indicates that the broad form of the multiline predominates after five flashes. The Kok analysis confirms that all centres that form multiline, broad or narrow, in the one-flash sample also form multiline in subsequent cycles of the enzyme, that is, both forms of the signal turn over efficiently by single flash advancement.

Fig. 3B shows the oscillation of the S_2 state multiline with flash number in samples containing MeOH, fresh and those stored for 16 weeks. In these samples there is a fraction of centres that produce a multiline on the first flash, which is not stable upon storage (i.e. the narrow form). On the fifth flash, however, *nearly all* centres produce a stable (broad) form of the multiline. At some point in the first enzymatic cycle, centres convert from a capacity to display the narrow form to displaying the broad S_2 state multiline. Reflashing the stored samples after a brief period of dark-adaptation, restored the multiline to its original intensity (data not shown). The experiment was repeated on MeOH-containing five-flash samples stored for 6 months. Of four samples, three lost between 0% and 8% of the signal intensity upon long-term storage and only one sample lost a substantial amount of signal upon storage (i.e. the narrow

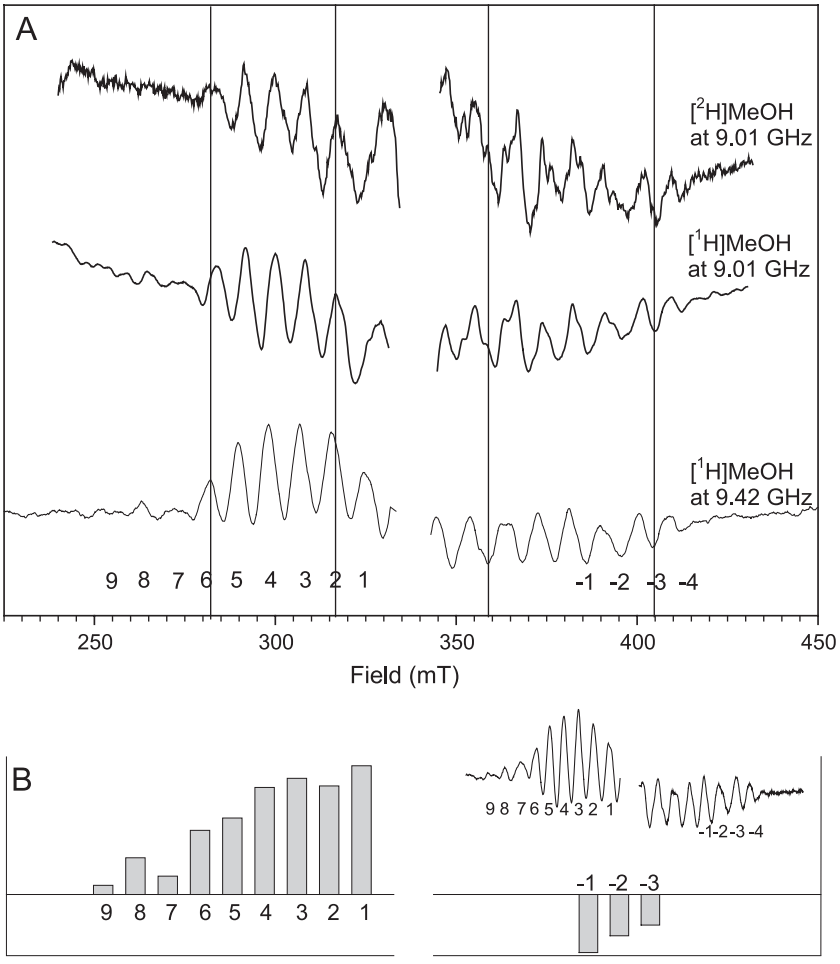

Fig. 4. (A) Alignment of narrow multiline spectra seen in the presence of methanol (~ 1 M) obtained from studies at two different X-band spectrometer frequencies (as indicated). The 9.01 GHz spectra are from the results of Evans et al. [10] and are the differences between cryo-stored and freshly re-illuminated PSII samples. The 9.42 GHz spectrum is from Fig. 2B. The field displacement necessary to give best overall alignment at 9.42 GHz corresponds to an apparent g-value of 1.85 ± 0.05. The modulation amplitude is 1.6 mT for the 9.01 GHz spectra and 2.0 mT for the 9.42 GHz spectrum. The upfield region in particular shows significantly more super-hyperfine resolution in the presence of [^{2}H]MeOH. (B) A partial 'stick spectrum' (see text) of the multiline component resistant to NIR turnover to the $g = 4.1$ state, from the results of Boussac et al. [2]. This is aligned with the spectra in panel A on the basis of the peak identifications given in Ref. [2].

form). We conclude that the broad form of the multiline signal is favoured in samples containing EtOH and also appears after five flashes in samples containing MeOH, i.e. on the second turnover of the enzyme.

3.3. CW characterisation of narrow multiline signal

The narrow multiline form was first identified by Evans et al. [10] based on its decay kinetics during cryo-storage and association with ESEEM detectable small-molecule species. Fig. 4A shows a comparison of spectra obtained at University College London (UCL) and Australian National University (ANU), as differences between PSII samples freshly advanced to S_2 by flash and then cryo-stored, or cryo-stored and re-illuminated at 200 K. MeOH is present ($\sim$ 1 M) in all cases, with both $^2H_3$ and $^1H_3$ methyl additions in the UCL data.

The signal is reproducible. It contains about 16 well-resolved peaks with a width of $\sim$ 130 mT, compared to the spectral width of the conventionally observed broad multiline of about 195 mT [15]. Small peaks to lower field of the sixth clearly resolved downfield peak may be present, but this is currently not certain.

Although the same modulation amplitude (1.6 mT) was used in the studies carried out at UCL, the [^{2}H] MeOH-containing spectrum has significantly more resolved super-hyperfine structure, than the [^{1}H] MeOH spectrum. This raises the possibility that methyl proton coupling from the MeOH may be substantial in the narrow multiline centre, sufficient to effect a discernable broadening of the hyperfine features in X-band CW spectra.

The UCL and ANU data were acquired at significantly different X-band frequencies ($\sim$ 9.01 and 9.42 GHz, respectively). This allows an estimate of the apparent *g*-value of the signal to be determined from the offset between the spectra at different frequencies (see Fig. 4). While this normally has only moderate precision for a frequency shift of $\sim$ 0.4 GHz, in the present instance the result is clear. The *g*-value of the narrow multiline centre is $\sim 1.85 \pm 0.05$. This is so different from the apparent *g*-value of the broad multiline species ($\sim$ 1.98 [1]) that it essentially eliminates the possibility that the narrow signal is some simple variant of the latter, with broadened wings lost in the signal to noise, etc.

3.4. ESEEM characterisation of narrow multiline signal

Fig. 5A shows frequency domain ESEEM spectra obtained at various field positions in the multiline envelope

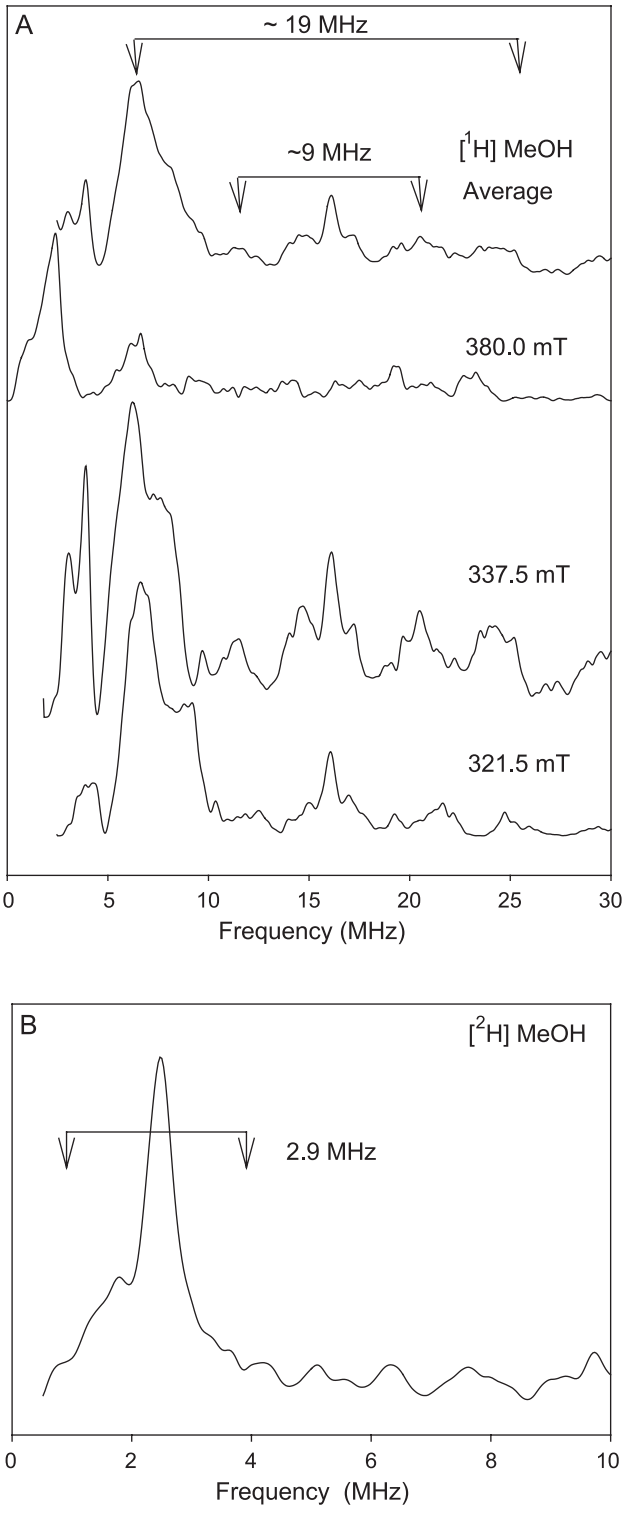

Fig. 5. (A) Frequency domain three-pulse ^{1}H modulation spectra from the multiline signal for [^{1}H] MeOH treated PSII membranes in the S_2 state (as in Ref. [10]). Spectra from three field positions across the multiline envelope were aligned at a common ^{1}H Larmor frequency (16.2 MHz) and averaged (thereby smearing out non-proton couplings). Two groups of quasi-isotropic proton couplings are indicated on the averaged spectrum. Width of the intense low frequency peak in the averaged spectrum is due partly to overlapping, distributed N resonances from lower field spectra. (B) Frequency domain three pulse ^{2}H/^{1}H time domain divided spectra for [^{2}H] and [^{1}H]MeOH-treated PSII membranes, aligned at the common Larmor frequency (2.48 MHz). Data from Ref. [10] are ^{2}H Larmor aligned and averaged, analogous to (A). For freshly illuminated samples, the intensity distribution of the ^{2}H modulations from [^{2}H]MeOH across the multiline envelope, is very similar to that for ^2H_2O [11]. Magnitudes of the larger ^{2}H coupling expected from the ^{1}H couplings in (A) is indicated. Spectrometer conditions: as in Ref. [10].

from freshly illuminated S_2 state PSII samples with ^1H MeOH present (~ 1 M). These show an evident low frequency peak, which overlaps the usual ^{14}N resonances at observation fields below ~ 350 mT, but becomes separated at higher H_0 fields (>380 mT). This peak appears to move with H_0 as a proton resonance, which is confirmed when the spectra are aligned at a common ^1H Larmor frequency (16.2 MHz, see Fig. 5A). In spectra acquired at a position near the multiline envelope maximum, the low frequency ^1H peak has an evident high frequency partner, consistent with a strong, quasi isotropic proton coupling of ~ 19 MHz. In this region, at least one other strong ^1H coupling (~ 9 MHz) is also visible. The presence of such strong couplings (~ 0.5–0.7 mT) from MeOH would indeed be consistent with the difference in hyperfine structure resolution seen in Fig. 4A, between the [^2H]- and [^1H]MeOH-treated samples. These strong couplings are absent from the ESEEM of [^2H]MeOH-treated PSII, but the latter shows a ^2H frequency domain peak envelope shape consistent with couplings of the magnitudes expected from the ^1H samples (see Fig. 5B). The two strong ^1H proton couplings seen here from MeOH are much larger than the proton equivalent couplings inferred from the [^2H]MeOH studies of Force et al. [12] (2.92 and 1.33 MHz). This is also reflected in the substantially different widths of the corresponding frequency domain resonance envelopes (compare Fig. 5B with Fig. 7 from Ref. [12]).

3.5. Signal quantitation

We have shown above that in the presence of MeOH there appears to be additional intensity in the S_2 state multiline spectrum, which can be shown to be due to a narrower form of the multiline present after the first flash, but substantially gone after five flashes. Double integration of the multiline signal after the first flash in EtOH, MeOH and with no additions, show that there are a substantial number of additional centres turned over in the presence of MeOH. We compared the integrated signal intensity from the three treatments. We found that the EtOH multiline intensity was only about 60% of the MeOH spectrum, and the sample with no addition, about 56% (Table 1). The oscillation behaviour of all signals with flash number were studied and could be fitted using similar Kok parameters, with the ratio of S_2 multiline centres observed after five flashes to that after one flash being about 60%, for each sample type. As the behaviour is approximately the same in each set of samples, the conclusion is that this difference is maintained; in the presence of MeOH more centres are turned over than otherwise. The difference is maintained over two turnovers. Continuous oxygen evolution measurements do not bring out such variations, although samples containing MeOH at these concentrations (< 1 M) often have slightly elevated oxygen evolution (~ 15%). It is may be that the observed differences vanish after several turnovers of the enzyme, which is why the large difference is not observed in the oxygen evolution measurements.

4. Discussion

Data from a number of groups have suggested that the multiline is not always a homogeneous species [3,5,6,10,11]. The clearest indications of this to date probably came from the differential susceptibility of PSII centres to NIR-induced conversion of the multiline to the $g = 4.1$ signal, seen by Boussac et al. [2] and the identification of the narrow multiline form on the basis of small molecule accessibility, by Evans et al. [10]. Boussac found that the lowest sensitivity to NIR conversion was when MeOH was present, and this NIR insensitive population of centres exhibited a narrower form of the multiline signal. On the other hand, EtOH containing samples were largely NIR sensitive. Boussac did not present a spectrum of the NIR insensitive component in his studies, but it is interesting to compare a ‘stick pattern’ for multiline peak positions showing relative NIR insensitivity derived from Boussac’s data, with the narrow multiline identified here. This is shown in Fig. 4B, and was obtained from Fig. 3 in Ref. [2], assuming simply that the maximum NIR turnover sensitivity seen corresponded to the broad multiline component in those samples. Although this is somewhat arbitrary, the comparison between the stick intensities and the spectra in Fig. 4A is suggestive of essentially the same species being involved in both cases. A similar heterogeneity in susceptibility to NIR was also found by Nugent et al. [16] in samples exposed to low temperature turnover to generate S_1 state ‘split radical’ signal. About half of the centres were not responsive to NIR turnover, and could form a split radical by ~ 10 K illumination and then a modified multiline signal by 77 K illumination. The other half the centres form the $g = 4.1$ signal in the presence of NIR.

Evans et al. [10] observed modulation due to ^2H from [^2H]MeOH in fresh PSII samples. This modulation decayed after a period of 3–4 weeks storage at 77 K. The modulation was found to be associated with a narrower form of the multiline. This result indicates that the component which gives rise to the modified multiline is exposed to the solvent. They obtained similar results using ^2H_2O [11], but here the decay was more rapid. Hence, they concluded that MeOH is not required for the formation of the narrow multiline signal, but access to the solvent phase is.

In ESEEM experiments using ^2H labelled EtOH and MeOH, Force et al. [12] found that both EtOH and MeOH bind in the immediate environment of the Mn cluster. Through point dipole analyses they reported that the methyl protons in MeOH are situated between 2.9 and 3.7 Å from the Mn and EtOH has a closest approach of 3.3 Å. They concluded that either of these small alcohols approach close enough to be a ligand.

The studies presented here allow us to identify features of the functional S_2 multiline state and differentiate between the effects of the above two alcohols, in a number of additional ways.

The functional S_2 state of the Mn cluster can exhibit *two* distinct forms of multiline signal. One is the well-known (broad) form, with ~ 20 lines and maximum total width of the hyperfine pattern of ~ 195 mT [15]. The other is a much narrower signal, with about 16 resolved lines and a width of ~ 130 mT. The central hyperfine line spacing is very similar, ~ 9.0 mT, in both cases and the patterns overlay essentially in registration at X-band. However, identification of the narrow form in difference spectra is *not* dependent simply on cancellation of features near the edges of the hyperfine multiline pattern, where signal to noise is always more challenging. Rather, multi-frequency studies show that the narrow form has an average *g*-value *so* different from the broad form, that the distinction is unambiguous.

The presence of MeOH promotes a configuration of the Mn cluster that gives rise to the narrow form of the multiline after one flash or ~ 200 K illumination, as observed by the decay of the signal upon sample storage. On the second turnover of the enzyme, virtually all centres give rise to the broader form of the multiline signal and signal intensity is therefore not lost upon storage. There is a conversion of the centres giving rise to the narrow form of multiline on the first turnover of the enzyme, to the broader form on subsequent turnovers. EtOH samples and those without addition, lose very little signal intensity upon storage and are therefore predominantly in the broad form. The broad form is probably the physiologically relevant form as it is favoured in samples containing EtOH or no additions and also formed upon multiple turnovers of the enzyme. It must be stressed however, that all centres which accept a first quantum of light cycle through the oxidation states and produce oxygen.

It appears that on the first turnover of the enzyme MeOH binds to the OEC in such a manner that the multiline hyperfine pattern and *g*-value are altered. This effect is dramatic in the case of the narrow multiline form, but is subtly present also in the broad form seen in the presence of MeOH. The latter is slightly different, particularly upfield, from the signal pattern seen in the presence of EtOH or with no alcohol additions (Figs. 1A, 2B and 3A). In addition the Q-band data (Fig. 1B) show that when both broad and narrow forms are present in MeOH samples, only the broad form gives a recognisable hyperfine pattern at Q-band. This exhibits some shifting of peak positions, probably suggesting a slight alteration in the *g*-anisotropy, but no major change in the average *g*-value from that of non-MeOH samples. The narrow signal appears not to be resolved at Q-band, at all. This is consistent with the relative total signal amplitudes seen at X-band for samples with and without MeOH (Fig. 1A) and the fact that the resolved multiline intensity in the MeOH sample at Q-band is no greater than that for the EtOH sample (Fig. 1B). It is possible that the large negative displacement in the upfield region of the Q-band MeOH sample is due to an underlying, unresolved component with $g < 1.9$, presumably arising from the narrow multiline species. If the average *g*-value of the latter is only ~ 1.85, then the centre almost certainly has a relatively large *g*-anisotropy, which could be sufficient to smear the signal envelope at Q-band. A similar effect is seen in mixed valence Mn dimers with significant *g*-anisotropy [17]. When the Q-band spectra are considered in the light of the storage loss data from Table 1, it appears reasonable to propose that most of the centres that initially form the narrow multiline in the presence of MeOH, do not turn over *at all* in the absence of MeOH (under the experimental conditions used for the EPR samples, at least). The small alcohol somehow activates centres that are functionally 'tardy'.

The above observations in the presence of MeOH make it a more likely candidate for direct ligation to the Mn than any other small alcohol species. The binding however is most likely at a site separate to the water binding site. This interpretation, although not conclusive, is the simplest in terms of the current data, given that O_2 activity is at or above control levels in the presence of up to ~ 3 M MeOH (see Ref. [12]). This is ~ 30 times the apparent binding K_d for MeOH to the multiline centre, as determined in S_2. Although similar data are not available for the S_3 state, it is known that the slower exchanging (presumably higher affinity) substrate water binding site exhibits the same exchange kinetics in both the S_2 and S_3 states [18]. Experiments to explore the effects of alcohols on substrate water exchange kinetics are planned.

The MeOH-induced pattern changes we associate with the broad multiline formed in the presence of this alcohol persist into the second turnover, as observed after five flashes at Q-band. It can persist in samples stored for a year at 77 K. Three in four of such samples studied after five flashes at X-band lost very little signal intensity upon storage, and were predominantly in the broad form. One sample lost some signal intensity, which highlights the fact that the effects we report here are trends, rather than rigorously reproducible outcomes. Our experience to date is that the presence of MeOH *predisposes* PSII samples to form the narrow multiline signal in a substantial portion of the centres on the initial passage to the S_2 state and that *generally* they have reverted to the broad form after one turnover. It is clear however, that factors which we have yet to identify, play a role. For instance, MeOH is not *necessary* to induce a narrow, solvent-accessible fast-decaying multiline species in a significant fraction of centres, from the ^2H_2O binding seen by ESEEM in MeOH-free systems [11]. Moreover, Boussac [5] was not able to clearly identify those conditions which influenced the extent of NIR-induced multiline to $g = 4.1$ turnover in PSII, although trends were noted.

Åhrling and Peterson [19] have previously shown that the MeOH-promoted S_0 state signal is present out to at least

eight flashes in a turnover series, so that MeOH effects on S_2 in terms of narrow multiline signal formation, are largely uncoupled from those on S_0. However, the samples that were used in the present studies showed relatively little S_0 hyperfine signal. It may be that S_0 multiline is favoured in samples that initially display less of the narrow form. We might expect only centres displaying the broad S_2 multiline to give rise to the S_0 state signal, as it first appears on the second turnover of the enzyme, where the broad form predominates. The situation is probably not explained so simply however, as EtOH-containing samples which are predominantly in the broad form, do not exhibit S_0 state multiline. Furthermore, Boussac [6] found the S_0 state signal unaffected by NIR light, i.e. displaying a similar behaviour to the S_2 state multiline in the presence of MeOH. The relationship between the S_0 multiline generation and the narrow form of the S_2 state multiline signal will be explored further.

Some further insight into these matters may come from what at first appears as a significant discrepancy between our data and that of Force et al. [12]. As noted above, the methyl ^{2}H couplings from MeOH seen by these authors in ESEEM were much smaller than those found here, either by ^{2}H or ^{1}H modulation. The weak ^{2}H couplings in Ref. [12] were interpreted as pure dipolar interactions between the methyl ^{2}H and the effective spin centre of the multiline cluster. In our case the strong proton interactions seen with [^{1}H]MeOH on the narrow multiline signal cannot be dipolar in origin. They must be mainly isotropic to be visible *at all* in the ESEEM powder pattern, although they may have some anisotropic component. The ESEEM frequencies for individual interactions then occur at approximately $|\nu_0 \pm A_{iso}/2|$ on the $\pm$ branches, where ν_0 is the ^{1}H Larmor frequency and A_{iso} is the isotropic component of the coupling. In Fig. 6, we reproduce the CW spectrum from Fig. 5b of Ref. [12], for their multiline signal generated in 1.0 M MeOH. This is seen to be *almost identical*, in shape and relative peak amplitudes, to the *second* turnover (five-flash) spectrum we observe with similar MeOH additions. As we have demonstrated above, the second-turnover type corresponds to the broad multiline seen in the presence of MeOH, although in the studies of Force et al, their multiline state was generated by a single turnover from S_1. We are thus lead to, what we regard as a satisfactory conclusion: both sets are valid observations and the methyl proton couplings from MeOH in the narrow multiline form are substantially larger than in the broad form. In both cases it appears however that MeOH binds to a Mn in the catalytic cluster, in a manner more specific than for any other small alcohol species so far studied. Factors that dictate whether and to what extent, the enzyme centres in S_2 exhibit the broad or the narrow form, remain as yet unclear.

The narrow form of the multiline exhibits about 16-line Mn hyperfine lines. Such signals are characteristic of dimeric Mn complexes and not generally of tetrameric Mn complexes with a ground $S = 1/2$ state [20], although theoretically predicted [7]. In these samples therefore at least a portion of centres would appear to have a multiline signal that is *dimer-like* and are fully functional in flash-advancement. After five flashes, these centres have converted to exhibit the most commonly encountered multiline signal, the broader form. It has previously been shown that PSII undergoes a light-adaptation process such that the relaxation behaviour of the Mn giving rise to the multiline, is altered on the second and subsequent turnovers of the enzyme [19,21]. This relaxation behaviour (not present on the first flash) disappears over a period of weeks in a five-flash sample stored on liquid N_2. In the samples studied here, we observed no change in signal intensity or shape over several weeks in the fifth flash sample. These two effects are therefore due to quite separate phenomena.

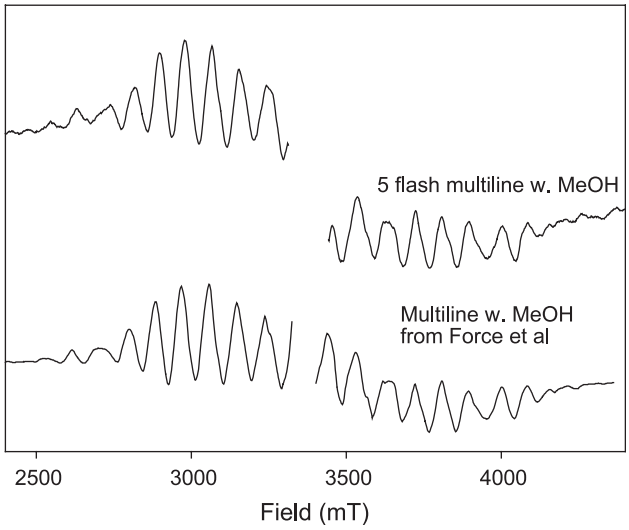

Fig. 6. Comparison of the ~1 M MeOH five-flash (second turnover) multiline spectrum, from Fig. 3A, with the 1.0 M MeOH multiline spectrum from Fig. 5 of Force et al. [12]. Both spectra are illuminated minus dark background. The spectrum from Ref. [12] was formed by continuous 200 K illumination (first turnover). See original reference for experimental details. The general shapes and relative intensities of the peaks suggest that this spectrum contains mostly the broad multiline species we see in the presence of MeOH.

4.1. A model for the MeOH effects

The broad and narrow multiline forms, although distinct, are clearly 'relatives' and show regions of hyperfine similarity, at least at X-band. Moreover and most importantly, both arise from functionally competent centres. It seems reasonable then that the general cluster geometry and formal oxidation states of the individual Mn ions are the same in both cases although the ligand environments are somewhat altered. Among the alcohols examined, only MeOH appears to bind in a specific manner, presumably due to its size. In the narrow multiline case at least, the magnitude of the methyl ^{1}H coupling requires that MeOH be a ligand to Mn, presumably a terminal ligand through O. As noted above, the binding site is unlikely to be a substrate water site.

Assuming this to be the case, the MeOH then binds either in a vacant site or by displacing a physiological ligand, perhaps a protein side chain. Its small size would be crucial in this.

In S_2, at least one Mn is in the III oxidation state [22]. Such a centre commonly experiences axial Jahn–Teller distortion, with either a $^5B_1$ or $^5A_1$ ground configuration. In the first case (axial ligation weak), the unpaired e_g electron is in the d_{z^2} orbital and $A_\perp > A_\parallel$ (in magnitude) for the Mn hyperfine interaction. In the second case the axial ligation is strong, the unpaired electron is in the $d_{x^2-y^2}$ orbital and the hyperfine anisotropy is reversed. All else being equal, switching from $^5B_1$ to $^5A_1$ configuration at one Mn III centre in the coupled cluster will narrow the readily discernable width of the resulting hyperfine powder pattern. Such a switch would be effected by strengthening the ligand interaction along the local axial direction for the Mn III ion.

Fig. 7 then summarises a simple model based on these ideas. We have previously suggested [15] that one Mn III centre (in the broad multiline form) is only five co-ordinate

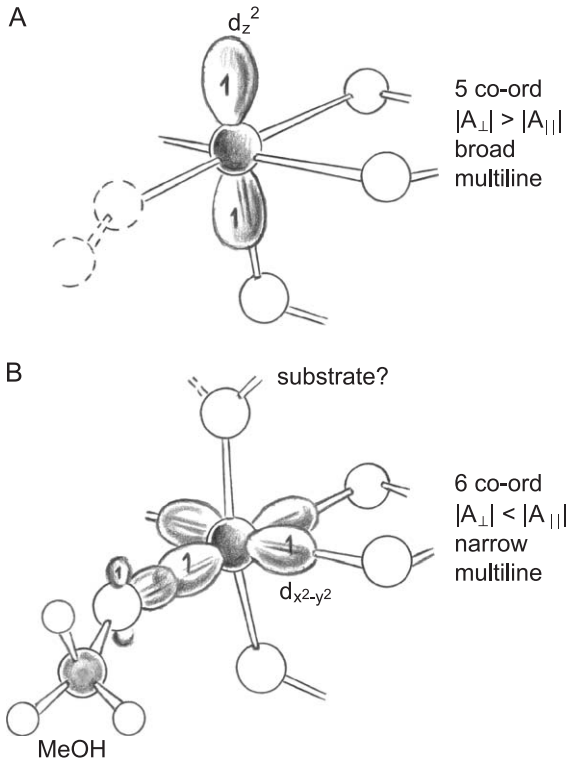

Fig. 7. Proposed model for the MeOH ligand binding position and Jahn–Teller interconversion occurring at a Mn III centre in the S_2 state of the coupled Mn cluster (see text). The suggested binding position for substrate water or hydroxide is along the axial *z*-direction, probably normal to the local di-μ-oxo plane. In the six co-ordinate (narrow multiline) configuration, the singly occupied e_g anti-bonding orbital is directed towards the MeOH ligand position, resulting in strong interaction with the methyl protons on MeOH. This position may possibly be occupied by a protein ligand in the native centre. In the five co-ordinate form, suggesting the physiologically normal configuration, the substrate ligand position is empty, or only distantly occupied. Then the methyl protons on MeOH see only weak interactions with the Mn d-electrons.

in S_2, with $A_{x,y} \gg A_z$ (Fig. 7A). This electronic configuration for Mn III is generally favoured in detailed simulations of the physiological multiline [7,23,24] and is, to our knowledge, the only configuration seen in Mn III model compounds. Evans et al. [11] have shown that the broad multiline form generally shows little ESEEM interaction with substrate water. If the electronic configuration of the Mn III centre 'flips' as a consequence of tight substrate (H_2O or OH^-) axial binding (Fig. 7B), then σ spin density from Mn will be directed towards the methoxy ligand, assumed bound in an equatorial position. This could induce some π spin polarisation on the methoxy O, resulting in classic β interactions with the methyl protons. Such interactions are mostly isotropic, as seen here, and the magnitude of the largest coupling, ~ 19 MHz, suggests ~ 15% net spin density in a p-orbital on the O. This is large, but if the hyperfine projection coefficient for the Mn III in the coupled system is ~ 2, as several models of the cluster coupling allow, e.g. [7,15,24,25] then the actual spin density is less than 10%, which is reasonable. Were this the case, it would also help rationalise the low g_{app} value for the narrow multiline. So large a deviation from 2.00, as seen, is unlikely to be a consequence of contributions from the Mn IV ions in the cluster. However, if the intrinsic Mn III ion contribution to the total *g*-value is doubled, as a consequence of the projection term, this might explain the large shift in g_{app} between the narrow and broad multiline forms.

We would interpret the results of Force et al. [12] to mean that MeOH may also bind, presumably in the same position, in the broad ($^5B_1$) configuration (Fig. 7A). Now there is no σ overlap with occupied Mn orbitals and if the geometry is unfavourable for π overlap (not unlikely in low symmetry), then only dipolar interaction between the Mn spin and the methyl protons is possible, as seen. The 'driving force' for the Jahn–Teller interconversion is the axial approach of the substrate O, the presence or absence of MeOH apparently being secondary. Evans et al. [10] see no ESEEM interaction of [^{2}H]MeOH with the broad multiline *at all*, while a species of narrow multiline can occur in the absence of MeOH [5,11]. As we have noted above, there are clearly matters yet to be resolved which influence the Jahn–Teller balance posited here. These may be related to variations in general solvent accessibility to the catalytic region, possibly reflecting variations in protein 'tightness' or matrix integrity. The latter could be turnover-dependent.

An alternative explanation for the difference in MeOH methyl proton couplings seen between the broad and narrow multiline forms is that the spin density on the ligating Mn III changes (e.g. due to a change in exchange interactions within the multiline cluster), without an alteration of the Mn III electronic structure. While this cannot be formally excluded, we regard it as unlikely. In the studies of Randall et al. [26], in which d_3-MeOH interaction with the Mn III ion in a Mn III–IV model dimer was examined by ESEEM, the deuteron coupling was found to be mainly dipolar and ~ twice the magnitude of that seen for d_3-MeOH interaction with the

multiline centre in PSII [12]. In the former instance, the Mn III configuration was $^5B_1$ and the alcohol binding was axial, along the extended Jahn–Teller axis (i.e. the *z* axis in Fig. 7). The spin density on the Mn III centre in the model compound is 'maximal' (i.e. the spin projection factor is ~ 2) and could not plausibly be higher for any ion in the photosystem cluster [7]. However, we observe MeOH methyl proton couplings to the multiline, in the narrow form, that are at least *three times* the dipolar interactions seen in the model dimer and more than *five times* those seen in the broad multiline form. The only physically reasonable explanation we can offer is that a significant change in the Mn III σ spin density directed *towards* the ligating MeOH occurs on transition from the narrow to broad multiline forms, which suggests a change in the d orbital electronic configuration. The fact that this may be triggered by a stronger, axial ligation of a substrate water molecule is supported by our very recent observation that interaction of O^{17} labelled water with the S_2 state Mn cluster is observable by ESEEM only on the narrow multiline form [27]. The MeOH interaction in the narrow multiline is somewhat analogous to that in the model dimer above, in that the alcohol ligates along the occupied e_g orbital direction (d_{z^2} in the latter case). However, the effect is attenuated in the model dimer, due presumably to the axial elongation known to occur along the Mn–OH CH_3 bond in that system [28].

It may at first seem implausible that a change such as we have suggested, even confined to one Mn centre, could have no apparent effect on the catalytic function of the site. There is one circumstance however where this could readily be possible—viz., if the structural lability in S_2 simply anticipated a change that was *going to occur anyway* (e.g. in S_3). It is interesting that the two groups who have studied the functional Mn cluster structure by EXAFS, through the S states, reached somewhat different interpretations of the $S_2 \rightarrow S_3$ turnover. Liang et al. [29] concluded that the principal change involved a substantial lengthening of one Mn–Mn 2.7 Å vector. Dau et al. [30] found however that in addition to this effect, there was a detectable *increase* in the first shell ligation to Mn (Mn–O,N). A simple hypothesis then assumes that the tight axial substrate binding described above *must always* occur in S_3 (if it has not already happened), as part of the normal catalytic function. The process might accompany oxidation of a bridge μ-oxo in one dimer, as proposed in Ref. [29].

5. Conclusion

Here we have shown that two very distinct forms of S_2 multiline signal may be identified and that the presence of MeOH favours a narrow form of the signal on the first turnover of the enzyme. This effect is removed upon subsequent turnovers and the broad form of the multiline predominates. We assume therefore that the broad form, which represents centres with limited solvent access, is the physiologically relevant form. In all three treatments examined here it is possible to get some mixture of the two forms. MeOH itself probably binds directly to the Mn cluster in both forms, and remains bound.

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